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Occurrence of aflatoxin in three maize (*Zea mays* L.) hybrids over 5 years in Northern Mississippi

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Abstract Aflatoxins are produced as secondary metabolites under conducive climatic conditions by *Aspergillus flavus.* The incidence of aflatoxin varies with environmental conditions, genotype, and location. An expanded understanding of the interaction of the plant, fungus, and weather conditions is needed to further elucidate the field infection process of maize by *A. flavus* and subsequent aflatoxin contamination. One of the problems in evaluating maize hybrids for resistance to kernel infection and aflatoxin contamination is identifying a time period and environmental conditions that are most advantageous. Three maize genotypes (Pioneer Brand 3223, Mo18W × Mp313E, and Mp313E × Mp420) were evaluated from 1998 to 2002 in response to *A. flavus* inoculation and aflatoxin contamination and weather conditions favorable for aflatoxin contamination were identified. The highest aflatoxin levels were observed in 1998 and 2000 (1186 and 901 ng g^{-1}; $P < 0.0001$); while the lowest levels were detected in 1999 (39 ng g^{-1}). Pioneer 3223 had significantly higher levels (1198 ng g^{-1}) than Mp313E × Mp420 (205 ng g^{-1}), and Mo18W × Mp313E (161 ng g^{-1}; $P < 0.0001$). The hybrids had six weather-related variables in common that were positively correlated with aflatoxin accumulation. Four of these occurred during 65–85 days after planting and were temperature-related. These results suggest that regardless of the hybrid's maturity or physiological development, the time from 65 to 85 days after planting may be indicative of a period of stress which leads to greater aflatoxin accumulation at harvest.

Keywords Aflatoxin · *Aspergillus flavus* · Environment · Maize

Introduction

Mycotoxins are considered to be among the most significant food contaminants because of their negative impact on public health, food security, and the national economy of many countries. They affect a wide range of agricultural products, including cereals, nuts, and oilseeds. Mycotoxin contamination of susceptible commodities occurs as a result of environmental conditions in the field as well as improper harvesting, storage, and processing operations [1–3]. Mycotoxins may be carcinogenic, mutagenic, teratogenic, and immunosuppressive. It has been estimated that annual losses in the USA and Canada, arising from the impact of mycotoxins on the feed and livestock industries, are of the order of $5 billion. In developing countries, where the food staples are susceptible to contamination, it is likely that significant additional losses occur within the human

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population because of morbidity and premature death associated with the consumption of mycotoxins [1].

Aflatoxins are an important group of mycotoxins that are produced as secondary metabolites under conducive climatic conditions by the fungi *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare [3]. Because of the established ability of aflatoxin to induce animal diseases, particularly liver cancer in human, aflatoxins are the most widely studied mycotoxins. More than 50 countries have established or proposed regulations for controlling aflatoxins in foods and feeds [4–6]. The U.S. Food and Drug Administration [7] has set a tolerance level of 20 ng g^{-1} for aflatoxin B_1 in maize (*Zea mays* L.). Grain that exceeds that level cannot be shipped through interstate commerce. Maximum tolerance levels differ greatly among countries [8]. As of 2003, 61 countries regulated aflatoxin B1 in foodstuffs [9].

Preharvest aflatoxin contamination is one of the main limitations of maize production in the humid and warm regions of the southern United States [10, 11]. In 1977 and 1998, Mississippi had severe problems with aflatoxin-contaminated maize [2]. In 1998, aflatoxin contamination resulted in $85–100 million in losses to maize producers in Texas, Louisiana, and Mississippi. The combined crop loss due to aflatoxin epidemics in the southern USA during 1988, 1989, 1995, 1996, and 1998 surpassed $1 billion [10]. This phenomenon has spurred research to identify factors that contribute to infection by *A. flavus*. The best strategy for aflatoxin control is to limit the amount in the developing maize crop. Maintaining good cultural and management practices that promote good crop health can reduce preharvest mycotoxin contamination [3, 4, 6, 10, 12]. However, no control strategy is completely effective when environmental conditions are extremely favorable for growth of the fungus [12]. Genetic variations in aflatoxin resistance are found in maize. Sources of resistance include inbred lines Mo18W, Mp313E, Mp420, Mp715, Mp717, Tex6, and population GT-MAS:gk [10, 13–18]. However, many of these lack acceptable agronomic performance that preclude their direct use in commercial hybrids [10].

The incidence and severity of ear rots and association with mycotoxins varies with environmental conditions, genotype and location [19]. The interactions between plants and pathogens can be shifted to favor either plant or pathogen by small changes in the environment, primarily temperature and plant nutrition [20]. Several studies have shown relatively large coefficients of variation that reflect the environmental impact on aflatoxin production [10]. Aflatoxin becomes more prevalent during drought stress because low rainfall and high temperatures encourage the growth and survival of the molds that produce the toxins, particularly during the silking to late dough stage of grain development. Generally, irrigated maize will have substantially lower levels of aflatoxin than non-irrigated maize, although several studies have shown that irrigation does not always reduce aflatoxin. In addition, aflatoxin levels tend to be higher in irrigated maize during drought years than nondrought years [5, 11].

Alleviation of environmental stress has been viewed as a major component of resistance to aflatoxin contamination and should be monitored throughout the life of the plant [21]. Adverse soil moisture and temperature conditions in combination with nutrient deficiencies, diseases, insects, and weeds interact to create many different kinds of crop stress [22]; thus, it is difficult to separate the effects of environmental stress. Most temperature stress conditions occur on high atmospheric-moisture demand days (HAMD), i.e., days when the daily mean temperature is above 25°C and daily maximum is above 35°C, regardless of soil moisture conditions [22].

Separating the effects of environmental stresses on aflatoxin production is difficult. The stage of plant development can affect the resistance or susceptibility to the pathogen [20]. Stress in the period from planting to seedling emergence is usually related to soil temperature, soil moisture, soil aeration, or an interaction between them; however, the plant's moisture requirement is very low. It has been suggested that moderate moisture stress during this period encourages early-season root growth, which could prove beneficial later if moisture supplies become limited.

Relationships between weather and yield are more significant in the late vegetative growth stages, i.e., the 3–4 week period prior to silking [22]. Widstrom et al. [23] conducted studies in Georgia that reveal significant correlations between aflatoxin content and maximum and minimum daily temperature, and net daily evaporation during the 20–40-day and 40–60-day periods following full silk. These two time periods span early and late post-inoculation phases of aflatoxin development.

The Midsouth's climatic conditions dictate that aflatoxin potential will continue to threaten maize producers until control measures are identified. Aflatoxin problems have historically developed during the years with severe high-temperature stress, particularly when coupled with water deficiency, insect ear, and stalk damage [2]. A great concern of many researchers is identifying a time period and environmental conditions that should be monitored in investigating the interaction between the host plant and the pathogen. The objectives of this article were to evaluate how three different maize genotypes (Pioneer Brand 3223, Mo18W × Mp313E, and Mp313E × Mp420) to *A. flavus* inoculation and subsequent aflatoxin contamination from 1998 to 2002; and to determine the weather conditions that were conducive to *Aspergillus* infestation and aflatoxin contamination.

Materials and methods

Growing conditions

One commercial maize hybrid (Pioneer Brand 3223) and two aflatoxin-resistant hybrids (Mo18W × Mp313E and Mp313E × Mp420) were at the R.R. Foil Plant Science Farm, Mississippi State, MS from 1998 to 2002. The hybrids were grown in a randomized complete block design with five replications. Individual plots consisted of a single row, 5.1 m in length, spaced 0.96 m apart, and thinned to 20 plants. Plots received supplemental irrigation during each growing season to limit drought stress. The planting dates for each year are listed as follows: 4 May 1998; 7 April 1999; 26 April 2000; 19 April 2001; and 1 May 2002 (Table 1). Other dates relevant to this analysis are also provided in Table 1.

Inoculation and aflatoxin analysis

The inoculum was produced by growing *A. flavus* isolate NRRL 3357 on sterile maize cob grits as described by Windham and Williams [5]. The primary ear of each plant was side-needle inoculated with 3.4 ml of a conidial suspension (10^{-6} conidia/ml) at 7 days after midsilk (DAMS; 50% of the plants in a plot had emerged silks) [24]. All ears were inoculated to minimize variation encountered in studies that rely on natural infection [25]. The ears were harvested 63 DAMS, dried at 38°C for 7 days, and machine-shelled. The grain from individual plots was thoroughly mixed and ground using a Romer mill (Union, MO). Aflatoxin analyses were performed on 50-g subsamples from each plot using the Vicam Aflatest (Watertown, MA), as previously described by Windham and Williams [5].

Environmental data

Environmental data were collected for several time periods spanning the period from planting to harvest in this study (Fig. 1): (A) the vegetative phase, the period from planting until 2 weeks before silking; (B) the silking bracket, 2 weeks before silking until 2 weeks after silking; (C) the grain filling period from 2 weeks after silking until harvest; (D) the period from 65 to 85 days after planting which has been indicated to be the most susceptible time of infection [21, 26]; (E) the times from 20 to 40 days and (F) 40–60 days after silking [23]; and (G) the entire growing season from planting to harvest. For each of these time periods, six environmental factors were selected for study. These included (1) growing degree units (GDU), calculated as [(daily low + daily high)/2] – 10°C, with the modifications that

Table 1 Planting, silking, and harvest dates for each year along with average aflatoxin concentrations

Year	Planting date[a]	Silking dates	Harvest dates	Average aflatoxin[b]
1998	May 4 (124)	June 29–July 20 (180–201)	August 31–September 21 (243–264)	6.1 a
1999	April 7 (97)	June 21–July 7 (172–188)	August 23–September 8 (235–251)	2.7 d
2000	April 26 (117)	June 23–July 10 (175–192)	August 25–September 11 (238–255)	5.8 ab
2001	April 19 (109)	June 20–July 9 (171–190)	August 22–September 10 (234–253)	4.7 c
2002	May 1 (121)	July 1–15 (182–196)	September 2–16 (245–259)	5.4 b

[a] Numbers in the parentheses are the Julian Calendar Day

[b] Aflatoxin concentrations presented as log-transformed (ln(aflatoxin + 1)) values averaged over all three genotypes

Means followed by the same letter are not significantly different ($P < 0.05$).

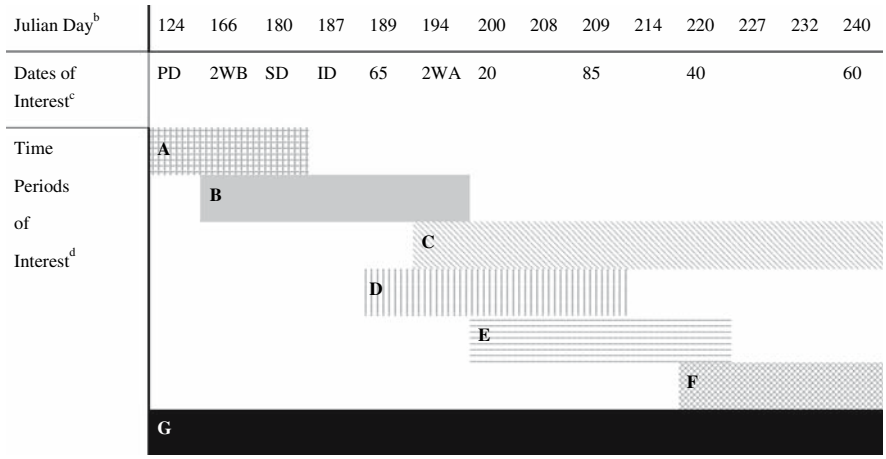

Fig. 1 Schematic[a] illustrating overlap of time periods spanning the period from planting to harvest for maize hybrid Pioneer 3223 in 1998. *Note*: [a]Not to scale. [b]Julian Calendar Day. [c]PD = Planting date; 2WB = 2 weeks before silking; SD = Silking date; ID = Inoculation date; 65 = 65 days after planting; 2WA = 2 weeks after silking; 20 = 20 days after silking; 85 = 85 days after planting; 40 = 40 days after silking; 60 = 60 days after silking; Harvest = harvest at 63 days after silking. [d]Time periods of interest: A = Vegetative growth from planting to silking; B = Silking bracket from 2 weeks before silking until 2 weeks after silking; C = Grain filling period from 2 weeks after silking until harvest; D = 65–85 days after planting which has been indicated as the most susceptible time of infection [21, 26]; E = 20–40 days after silking [23]; F = 40–60 days after silking [23]; G = Entire growing season from planting until harvest

if daily low <10°C = 10 and if the daily high >30°C = 30, temperatures below 10°C or above 30°C are typically ineffective for maize kernel development [27]; (2) rainfall accumulation (mm); (3) number of days (Max) the maximum temperature exceeded 30°C [28]; (4) the number of days (Min) the minimum temperature exceeded 22°C [29]; (5) pan evaporation (PAN), a measure of the evaporative power of the air [30]; and (6) HAMD, as defined by Shaw and Newman. [22], when the average temperature exceeds 25°C and the maximum temperature exceeds 35°C. Daily maximum and minimum temperatures, total rainfall, and pan evaporation data were obtained from the Mississippi Agricultural and Forestry Experiment Station (MAFES) station at the R.R. Foil Plant Science Farm, Mississippi State, MS.

Statistical analysis

Aflatoxin concentrations were log-transformed (ln (aflatoxin + 1)) to stabilize variance of the data. The aflatoxin concentration data presented are the log-transformed means. Aflatoxin means were compared using the least significant ratio (LSR). The mean values of other data were compared using Fisher's protected least significant difference (LSD). The significance of differences between genotypes and years for aflatoxin was determined by *F*-test, and the treatment means were compared by LSD at $P = 0.05$. Means are based on five replications. Means in a column followed by the same lowercase letter do not differ at $P = 0.05$; means in a row followed by the same uppercase letter do not differ at $P = 0.05$.

The statistical analysis was performed in stages. Initially, comparisons were made evaluating hybrid response by year and between years by generalized least model (GLM) in SAS V8 (SAS Institute, Cary, NC, USA). Further analyses were conducted per individual hybrid because the hybrids are of different maturities. Spearman's correlation coefficients between aflatoxin and the weather related variables were examined using the Proc Corr Procedure in SAS V8. Prior to any correlation or multivariate analysis, all data were standardized to have a mean of 0 and a standard deviation equal to 1. Standardization of data is recommended when the measured variables are in completely different units [31].

Results

Aflatoxin analysis

The level of *A. flavus* infection and aflatoxin accumulation varied with years and locations and was

generally greater during years with periods of moisture stress [16]. Over the course of this 5-year period, there were significant differences in aflatoxin accumulation between the years ($P < 0.0001$; Table 1). As previously reported [10], record high aflatoxin levels were seen in 1998. The years 1998 and 2000 represented the highest overall average aflatoxin levels in the three lines used in this study (6.1 and 5.8, respectively). There were no significant differences in aflatoxin accumulation between the years 2000 and 2002 (5.4), though 2002 had significantly lower levels of aflatoxin accumulation than 1998. The year 1999 (2.7) represented the lowest levels of aflatoxin accumulation in this study. The year 2001 (4.7) was significantly different from each of the other years (Table 1). These results highlight some of the yearly variability which is encountered with aflatoxin accumulation in maize.

Overall, there were significant hybrid differences in aflatoxin accumulation ($P < 0.0001$; Table 2). During the span of these 5 years, Pioneer 3223 averaged 7.1 and had significantly higher levels of aflatoxin accumulation than Mp313E × Mp420 (5.1) and Mo18W × Mp313E (5.3). Overall, there were no significant differences between the hybrids Mp313E × Mp420 and Mo18W × Mp313E. There were significant hybrid by year interactions ($P < 0.0001$; Table 2). In 1998, 2000, and 2001, there were significant differences between Pioneer 3223 and the hybrids Mo18W × Mp313E and Mp313E × Mp420. In each of these years, there were greater levels of aflatoxin accumulation in Pioneer 3223 and there were no significant differences between the other hybrids. In the years 1999 and 2002, there were no significant differences in aflatoxin accumulation between any of the lines.

The hybrids accumulated different levels of aflatoxin over the years of this study. Pioneer 3223 accumulated similar levels of aflatoxin in the years 2000, 2001, and 2002. In 1998, significantly greater levels of aflatoxin were detected while in 1999, the levels were significantly lower. Mo18W × Mp313E performed similarly in 1998, 2000, and 2002 and accumulated lower levels of aflatoxin in 1999 and 2001. Mp313E × Mp420 accumulated its highest levels of aflatoxin in 1998, 2000, and 2002, although the levels accumulated in 1999 and 2001 were not significantly different from accumulation in 1998 (Table 2).

Weather analysis

The relationship between the aflatoxin production and the environmental variables varied between the hybrids. Of all the weather-variables generated, 32 were significantly correlated with the aflatoxin accumulation in Pioneer 3223 and 29 were significantly correlated with aflatoxin accumulation in Mo18W × Mp313E. These occurred in each of the time periods of interest and were primarily related to GDU, MAX, MIN, HAMD, rainfall accumulation, and pan evaporation. Only seven weather-related variables were significantly correlated with aflatoxin production in Mp313E × Mp420. All seven were within the 65–85 DAP and the silking bracket time periods.

The hybrids had in common six weather-related variables with significant positive correlations to aflatoxin accumulation: HAMD during the silking bracket; and GDU, MAX, MIN, HAMD, and Pan within the 65–85 DAP time period (Table 3). The variables GDU, MAX, MIN, and HAMD are all indicators of temperature. These results indicate that there is a positive correlation between heat stress during the 65–85 DAP and aflatoxin accumulation at final harvest. With Pioneer 3223, the 65–85 DAP time period typically began 8–10 days after silking. With the later-maturing hybrids, Mo18W × Mp313E and Mp313E × Mp420, silking tended to begin around 85 DAP.

Discussion

The role of the weather, primarily moisture and temperature stress, in the preharvest contamination of maize with aflatoxin is well documented. Aflatoxin problems are more likely in Mississippi than in the U.S. Maize Belt, because the state's hot, humid climate is ideal for fungal growth [2]. Maize hybrids may be exposed to an increased risk of aflatoxin contamination when environmental stress, e.g., drought and high temperatures occur during the flowering and grain filling periods [4]. The crucial stage of maize plant development occurs during a 3-week period following full silk. It is probable that environmental conditions that induce plant stress also favor fungal development and/or aflatoxin synthesis. It has been reported that plant–fungus interactions during the period from silking to physiological

Table 2 Comparison of aflatoxin accumulation[a] between genotypes over 5 years

	Overall[b]	1998	1999	2000	2001	2002
Pioneer 3223[c]	7.1 a	7.6 aA	3.1 aC	6.6 aB	6.5 aB	6.5 aB
Mo18W × Mp313E	5.3 b	5.5 bA	2.5 aC	5.5 bA)	3.6 bBC	4.8 aAB
Mp313E × Mp420	5.1 b	4.2 bAB	2.3 aB	5.3 bA	3.8 bAB	4.6 aA

[a] Aflatoxin concentrations presented as log-transformed (ln(aflatoxin + 1)) values

[b] Differences in yearly hybrid aflatoxin accumulation. Means within a column followed by the same lowercase letter are not significantly different ($P \leq 0.05$)

[c] Differences in aflatoxin accumulation per hybrid over time. Means within a row followed by the same uppercase letter are not significantly different ($P = 0.05$)

Table 3 Spearman correlation factors between aflatoxin accumulation and six weather variables in three hybrids over 5 years

	GDU[a]	MAX[b]	MIN[c]	HAMD[d]	HAMD[e]	PAN[f]
Pioneer 3223	0.79**	0.45*	0.80**	0.62**	0.42*	0.78**
Mo18W × Mp313E	0.71**	0.66**	0.74**	0.69**	0.67**	0.51*
Mp313E × Mp420	0.51*	0.57**	0.47*	0.55*	0.45*	0.54*

[a] GDU = Growing degree units, calculated as [(daily low + daily high)/2] − 10°C, with the restrictions that if daily low <10 = 10 and if daily high >30 = 30 during the time period 65–85 days after planting

[b] Max = Number of days when the maximum temperature exceeded 30°C for the time period 65–85 days after planting

[c] Min = Number of days when the minimum temperature exceeded 22°C for the time period 65–85 days after planting

[d] HAMD = High atmospheric-moisture demand days = when the average temperature exceed 25°C and the maximum temperature exceeds 35°C for the period from 2 weeks before silking until 2 weeks after silking

[e] HAMD = High atmospheric-moisture demand days = when the average temperature exceed 25°C and the maximum temperature exceeds 35°C for the period 65–85 days after planting [22]

[f] PAN = Open pan evaporation (mm/day) for the period from 65–85 days after planting

[g] *, ** corresponds to significance level at $\alpha = 0.05$ and 0.01, respectively

maturity are important to aflatoxin contamination [26]. Widstrom et al. [23] suggest that the most vulnerable time for fungal penetration through wounds to achieve maximum aflatoxin concentration at harvest is approximately 20 days after full silk. In a review, Widstrom [21] states that the period from 65 to 85 days after planting is the most susceptible time for infection.

Regardless of the hybrid's maturity group or physiological growth stage, the time period from 65 to 85 days after planting may be indicative of a period of abiotic stress, particularly heat stress, which leads to greater accumulation of aflatoxin at harvest. The data proposes that the resistant hybrid Mp313E × Mp420 may be more capable of withstanding the environmental stresses that may trigger greater levels of aflatoxin accumulation, particularly during the period from 65 to 85 days after planting. Given the various environmental events that may occur during a growing season, this information could assist other researchers in evaluating changes in physiological development and/or gene expression during a specific time period that could lead to new insight on aflatoxin resistance in maize. An expanded understanding of the interaction of the plant, fungus, and weather is needed to further elucidate the field infection process of maize by *A. flavus* and subsequent aflatoxin contamination. This report emphasizes the importance of high temperatures during a select time period under field conditions and contributes to the understanding of regional environmental factors that may influence the growth of aflatoxigenic fungi.

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